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A Hepatitis B Virus Variant Found in the Sera of Immunised Children Induces a Conformational Change in the HBsAg "a" Determinant

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The emergence of variants in the outer envelope proteins of hepatitis B virus (HBV) are found among individuals vaccinated against HBV and asymptomatic carriers of the infection. For example, children in The Gambia vaccinated against hepatitis B may show serological evidence of breakthrough infections, particularly if anti-HBs antibodies induced by the vaccine are low in titre. A single-point mutation at nucleotide 421 of the S gene is associated with such breakthrough infections. In the present study, the antigenicity of variant HBV S protein expressed as HBsAg particles in a vaccinia virus expression system has been characterised using a panel of monoclonal antibodies directed against linear and conformational determinations of the S protein. A cellular ELISA procedure using expressed antigen in Vero cells revealed differences in reactivity using four of the six antibodies that had been raised against the *adw* subtype of HBV and recognise conformational epitopes in the *a* determinant. In two instances, an enhanced reactivity for the variant antigen was found, confirming that point mutations in the *a* determinant of the S protein between residues 139 and 147 may result in significant changes in conformation. These findings also demonstrate that there are distinct antigenic differences between the vaccine strains of HBsAg/*adw* subtype and the predominant HBsAg subtype circulating in West Africa. The implications of this work are that serodiagnosis of HBV infections may be unreliable in populations where there is a possibility of variant HBV infections emerging in the face of increasing herd immunity to HBV as a result of vaccination, particularly using monoclonal antibody-based diagnostic tests. Such variants may play a role in the maintenance of HBV infections in endemic regions. ***J. Med. Virol. 58:346–352, 1999.***

KEY WORDS: hepatitis B virus; antigenic variation; hepatitis B vaccines

INTRODUCTION

Universal immunisation of all newborns against hepatitis B virus (HBV) is being introduced into childhood immunisation programmes, especially in regions where the disease is endemic. However, there is evidence of breakthrough infections in 5% to 19% of those children vaccinated. Many of these have protective levels of antibodies against hepatitis B surface antigen (HBsAg), the major component of the viral envelope (Whittle et al., 1991; Fortuin et al., 1994). Up to 99% of the vaccine-induced antibody response is directed against the group-specific *a* antigenic determinant of HBsAg (Jilg et al., 1989), a response that is normally equated with immunological protection. The sequencing of viral DNA recovered from individuals with breakthrough infections has shown that there are amino acid substitutions within the immunodominant region of the *a* antigenic determinant, located between residues 137 and 149 (Howard, 1995a). One of these variant strains, detected in viral DNA isolated from children given hepatitis B immunoglobulin (HBIG) at the time of the first dose of vaccination, is located at residue 145 of the HBsAg and results in a glycine to arginine substitution (Carman et al., 1992; Harrison et al., 1992; Okamoto et al., 1992). Certain monoclonal antibodies against the native *a* antigenic determinants

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do not react with this particular variant antigen (Waters et al., 1992). Previously we have demonstrated that there are at least three epitopes within the *a* determinant region, and that vaccine-induced anti-*a* antibodies may differ in reactivity profiles to these epitopes compared to convalescent anti-*a* antibodies (Thanavala et al., 1986).

We reported previously the isolation from immunised children in The Gambia a unique variant strain of HBV DNA characterised by a critical lysine to glutamic acid substitution at residue 141 (Karthigesu et al., 1994; Fortuin et al., 1994). Of the three lysine residues located within the antigenic domain of HBsAg between residues 101 and 159, only the lysine 141 residue is known to be critical for the binding of anti-*a* antibodies as acylation of the free amino group abrogates antibody binding to HBsAg particles (Neurath et al., 1984; Neurath et al., 1990; Steward et al., 1993). In contrast to studies in children born to carrier mothers and given both vaccine and HBIG, the importance of this work is that breakthrough infections have been found in Gambian children receiving vaccine only. In a more recent study, approximately 8% of immunised children in The Gambia have evidence of breakthrough infections (Whittle et al., 1995).

In this study the hypothesis was investigated that a single amino acid change may significantly alter one or more conformational-dependent epitopes with the HBsAg *a* determinant, and that such changes may be defined by use of murine monoclonal antibodies directed principally against conformational epitopes. For this work we have developed an ELISA for use with cells infected with recombinant vaccinia virus expressing either wild-type HBsAg (*awy4* subtype), with the conserved lysine at residue at position 141, or the variant HBsAg particle with substituted glutamic acid at this position. Surprisingly, it was found that this single amino acid change results in enhanced antibody binding to some monoclonal antibodies raised against wild-type antigen, adding further evidence that there may be a spectrum of conformational epitopes within the *a* determinant. These findings of differences in reactivity of the wild-type and variant antigens have implications for the serodiagnosis and future vaccination strategies against hepatitis B.

MATERIALS AND METHODS

Monoclonal Antibodies to HBsAg

A panel of six mouse-derived ascitic fluids containing anti-HBs monoclonal antibodies, prepared using recombinant HBsAg of the *adw* subtype, was produced at the National Institute for Biological Standards and Control, U.K. The murine monoclonal antibodies were identified numerically as 1020, 1021, 1023, 1030, 1031, and 1044, respectively. Antibody RFHB1 was kindly provided by D.J. Waters, Imperial College, London.

Cells

CV-1 (African green monkey kidney fibroblast), 143 TK$^-$ and Vero cells (African green monkey kidney cell line) were all obtained from the American Type Tissue Culture Collection, Rockville, USA. Cells were grown in 1x Dulbecco's minimum essential medium (DMEM, Gibco BRL, Paisley, Scotland), 0.018% (w/v) sodium bicarbonate, 2 mM L-glutamine (Gibco BRL), 50 units penicillin and 50 μg streptomycin per milliliter (Gibco BRL). Growth medium included 10% (v/v) bovine foetal serum (FCS, Gibco BRL), while 2% FCS was added to the maintenance medium.

Plasma-Derived HBsAg

Plasma-derived HBsAg, subtype *adw* (a kind gift from Dr. R. Ellis, Merck and Co., USA), was used as a positive control antigen (HBsAg^{adw}) and for the initial titration and characterisation of the monoclonal antibodies.

Recombinant HBsAg

HBV DNA recovered from a chronic carrier which contained the conserved lysine at codon 141 of HBsAg, and a variant isolate from an immunised child with an asymptomatic breakthrough infection, were used in this study. The origin of these samples has been described (Karthigesu et al., 1994). The HBsAg was expressed using a vaccinia virus expression vector (Chakrabarti et al., 1985). Briefly, the entire 681-nucleotide S gene of each isolate, which had previously been cloned into pGEM-3Z plasmid (Promega, Madison, WI), was excised using the restriction enzymes *EcoR*I and *Pst*I (Pharmacia, Uppsala, Sweden). The ends were repaired using Klenow (Large Fragment of DNA polymerase I, BRL), and blunt-end ligated to the *Sma*I (Boehringer-Mannheim)-digested transfer vector pSC11, which directs the insertion of foreign genes together with the β-galactosidase (β-gal) gene, derived from *Escherichia coli,* into the thymidine kinase (TK) locus of the vaccinia virus genome. The entire S gene sequence insert within each of the two recombinant pSC11 plasmids was sequenced by thermocycle sequencing (*fmol* DNA Sequencing System, Promega) as described before (Karthigesu et al., 1994). The recombinant pSC11 constructs were transfected into vaccinia virus-infected CV-1 cell cultures and recombinant vaccinia viruses were selected using 143 TK$^-$ cells in the presence of 5-bromouridine (BUDR). The two recombinant clones selected were identified as vHBsAg^{ayw4} and vHBsAg^{E141}; these expressed HBsAg of the *ayw4* subtype with the conserved lysine 141 and the variant glutamic acid 141, respectively. The recombinant viruses were plaque-purified twice in 143 TK$^-$ cells, passaged twice in CV-1 cells and subsequently used as working seeds.

Immunoassays

Titration of monoclonal antibodies against plasma-derived HBsAg^{adw} by ELISA. The monoclonal antibodies were titrated using an antibody capture ELISA. Flat-bottomed microtitre ELISA plates (Immulon I, Nunc) were coated overnight at 4°C with 250 ng per well of plasma-derived HBsAg^{adw} in carbonate-bicarbonate buffer (0.015 M sodium carbonate, 0.035 M sodium hydrogen carbonate, 0.02% (w/v) so-

dium azide, pH 9.6) and then blocked for 2 hours at 37°C with 200 μl per well of 1% gelatin in PBS, pH 7.4. The wells of the microtitre plates were washed with PBS-T (0.05% Tween 20 in PBS, pH 7.4) between each step of the ELISA reaction. Fourfold dilutions of the monoclonal antibodies starting with dilution of $\log_{-10}$ 2.7 of the antibodies in diluent (0.25% gelatin in PBS-T) were added (50 μl) in triplicate, and reacted overnight at 4°C. Wells containing antibody diluent served as background controls. Unbound antibodies were washed away, and 25 μg of affinity-purified horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated goat anti-mouse IgG per 50 μl in antibody diluent was added to each well. The plates were incubated at 37°C for 1 hour. Subsequently each well was reacted with 20 μg per 50 μl of *o*-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis, MO) substrate in citrate phosphate buffer (540 mM disodium hydrogen phosphate, 27 mM citric acid, pH 5.0) containing 0.018% (v/v) hydrogen peroxide and reacted for 10 minutes at room temperature. The reactions were stopped with 25 μl of 2 M sulphuric acid, and the colorimetric reactions was read at $OD_{490\ nm}$ using an ELISA spectrophotometer (MR5000, Dynatech, Billinghurst, UK). The mean $OD_{490\ nm}$ value and standard deviation (SD) at each antibody dilution was determined, and used to generate a line graph with Excel 5 (Microsoft).

The differences in the reactivity of the monoclonal antibodies to HBsAg^{adw} were verified by testing five or six replicates of each antibody at a dilution of $\log_{-10}$ 5.1. The mean $OD_{490\ nm}$ and standard deviation (SD) were determined, and the significance of the differences in the mean values was ascertained using the single-tailed, unpaired Student's *t* test and the Instat2 programme (GraphPad Software, San Diego, CA).

Cellular-ELISA for titration of monoclonal antibodies against rHBsAg^{ayw4} and rHBsAg^{E141}. The two recombinant viruses, vHBsAg^{ayw4} and vHBsAg^{E141}, together with vaccinia virus devoid of any HBsAg inserts serving as the negative antigen control (vV), were titrated on Vero cells. Monolayers of 3-day-old cultures of Vero cells were infected with six replicates of twofold dilutions of the viruses in a dilution series from 10^{-3} to 2.048×10^{-6}. The cultures were incubated at 37°C with 5% carbon dioxide and examined daily for the presence of cytopathic effect (CPE). The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the Karber method (Irwin et al., 1939).

To confirm infection and expression of HBsAg, monolayer cultures of 1-day-old Vero cells in 96-well tissue culture microtitre plates (Nunc) were infected with varying dilutions of recombinant vHBsAg^{ayw4}, vHBsAg^{E141} and the vV control viruses. An immunoperoxidase assay was carried out 3 days postinfection as described previously (Russell et al., 1983) with 50 μl of a $\log_{-10}$ 3.3 dilution of monoclonal antibody 1030. The plates were examined for immunoperoxidase staining using an inverted microscope.

The cellular-ELISA was carried out using Vero cells seeded in flat-bottomed 96-well tissue culture plates (Corning Disposable Multiple Well Plates) at 4×10^4 to 1.3×10^5 cells per 100 μl per well. The recombinant viruses (vHBsAg^{ayw4}; vHBsAg^{E141}) and vaccinia virus control (vV) were diluted in maintenance DMEM to contain 4×10^2 $TCID_{50}$/ml, and were inoculated (100 μl per well) into 3- to 4-day-old monolayer cultures. At 3 days postinfection, infected cells were fixed for 10 minutes at room temperature with 100 μl per well of formaldehyde (10% [v/v] in 0.36% sodium dihydrogen phosphate, 0.65% disodium hydrogen orthophosphate) and washed three times using deionised water. Dilutions of the monoclonal antibodies were previously optimised to show the differences in reactivity of each antibody for the two recombinant HBsAg substrates. Before it was used in the cellular-ELISA, each antibody was diluted in the range of $\log_{-10}$ 2.3 to $\log_{-10}$ 6.5 in 10% FCS in carbonate bicarbonate buffer (pH 9.6), and added in triplicate (50 μl) to the fixed cells. Following overnight reaction at 4°C, the cells were washed three times and reacted, for 1 hour at 37°C, with 25 μg per 50 μl of HRP conjugated goat anti-mouse antibody diluted in PBS (pH 7.4) containing 10% FCS. The plates were rewashed and reacted with OPD (20 μg per 50 μl) for 10 minutes at room temperature. The reactions were stopped with 25 μl of 2 M sulphuric acid, and read at $OD_{490\ nm}$. The mean $OD_{490\ nm}$ values and the corresponding SD at each dilution were determined.

To ensure reproducibility, the concentration of the recombinant viruses in the inocula used for infection was confirmed by titration on Vero cells. Briefly, each inoculum was diluted fourfold and inoculated in triplicate into wells of a separate plate. The plate was examined for CPE following an incubation period of 3 days and the $TCID_{50}$ calculated as described above. Variations in the concentration of the inocula of the two vaccinia virus recombinants diluted to contain 4×10^2 $TCID_{50}$/ml did not vary by more than 1.6 $TCID_{50}$ (data not shown).

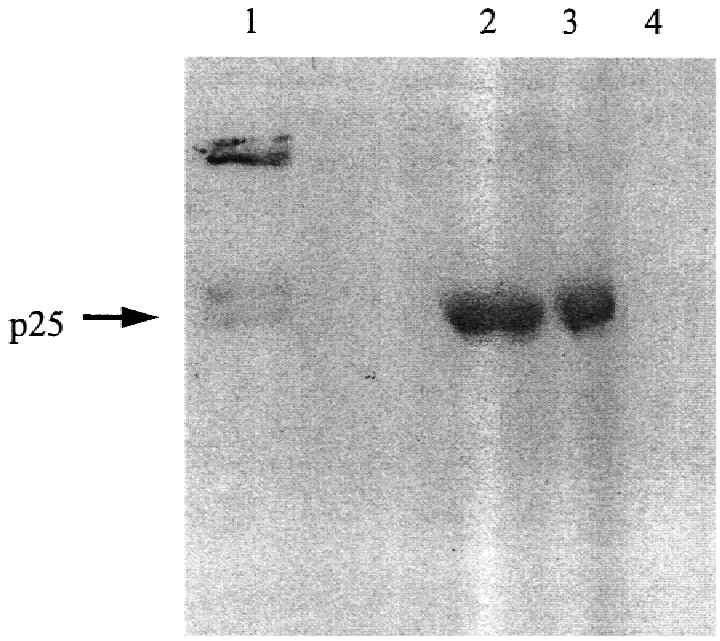
RESULTS

The potential effect of the lysine 141 to glutamic acid substitution on the antigenicity of the S protein was investigated by comparing the reactivity of a panel of monoclonal antibodies against expressed variant HBsAg and wild-type HBsAg from the same region. Comparisons were made also between the reactivities of the antibodies for plasma-derived HBsAg of subtype *adw* to determine to what extent this single-point mutation affected the common *a* determinant epitopes.

Preparation and Characterisation of the Vaccinia Virus Constructs

Two vaccinia virus constructs were prepared, the first expressing the wild-type antigen prevalent in The Gambia (vHBsAg^{ayw4}) and the second containing the S gene encoding for the S antigen variant (vHBsAg^{E141}). A comparison of the sequence of S gene inserts within vHBsAg^{ayw4} and vHBsAg^{E141} revealed four nucleotide differences. Adenosine was detected at nucleotide 421 within vHBsAg^{ayw4} indicating that the lysine 141 is

(a) Antigenic analysis of expressed HBsAg



(b) Sequence comparisons

	124														137	139											150
adw	C	T	T	P	A	Q	G	N	S	M	F	P	S	C	C	C	T	K	P	T	D	G	N	C	T	C	I
ayw4	C	T	T	**L**	A	Q	G	**T**	S	M	F	P	S	C	C	C	**S**	K	P	**S**	D	G	N	C	T	C	I
ayw4 mutant	C	T	T	**L**	A	Q	G	**T**	S	M	F	P	S	C	C	C	**S**	**E**	P	**S**	D	G	N	C	T	C	I

⇑

RFHBS1

Fig. 1. Vaccinia recombinant expression of HBsAg^{ayw4} and HBsAg^{E141}: **(a)** Immunoblotting of HBsAg particles using reference antibody RFHBS1 directed against linear epitopes located between amino acids 124–137 (Waters et al., 1991); *1,* HBsAg^{E141}; *2* and *3,* HBsAg^{ayw4}; *4,* vaccinia virus vector control. The HBsAg^{E141} sample shows evidence of incomplete reduction of the major S protein. **(b)** Comparison of amino acid sequences between residues 124 and 150 (Norder et al., 1993 and this study). Antibody RFHBS1 recognises an epitope within the sequence 124–139 (Waters et al., 1991).

conserved in this construct, as was found previously for the wild-type isolate (GenBank accession number L29017). In addition, isoleucine detected at codon 49 within vHBsAg^{E141} had been replaced by cysteine in the vHBsAg^{ayw4} strain. The nucleotide changes at positions 135 and 625 do not alter the alanine and leucine encoded at amino acid positions 45 and 209, respectively.

The reactivity of the expressed HBsAg proteins was measured in the first instance by ELISA using two reference monoclonal antibodies previously determined as recognising linear epitopes between residues 124–137 and 139–147, respectively (Waters et al., 1991). These data showed that there was a reduction in binding for the variant protein using the antibody directed against amino acids 137–149, the domain where the major *a* determinant epitopes are located (Fig. 1a). Immunoblotting confirmed that both antibodies bound the wild-type HBsAg^{ayw4} protein but not the variant HBsAg^{E141} (data not shown). The antibody directed against an epi-

TABLE I. Reactivity of Monoclonal Antibodies for Homologous ($HBsAg^{adw}$) and Heterologous ($HBsAg^{ayw4}$) Antigens as Measured by ELISA and Cellular ELISA, Respectively

	Solid phase substrate (SD)		
Mab	$HBsAg^{adw}$	$HBsAg^{ayw4}$	vV control
1020	1.28 ± 0.053[1]	1.18 ± 0.062	0.135 ± 0.007
1021	2.11 ± 0.071	0.46 ± 0.036	0.176 ± 0.007
1023	1.16 ± 0.097	1.08 ± 0.047	0.167 ± 0.016
1030	1.92 ± 0.019	1.00 ± 0.045	0.211 ± 0.004
1031	2.02 ± 0.058	1.54 ± 0.052	0.240 ± 0.052
1044	1.97 ± 0.058	1.54 ± 0.067	0.241 ± 0.108

[1]Mean $OD_{490\ nm}$ values from a minimum of five replicate reactions are shown.

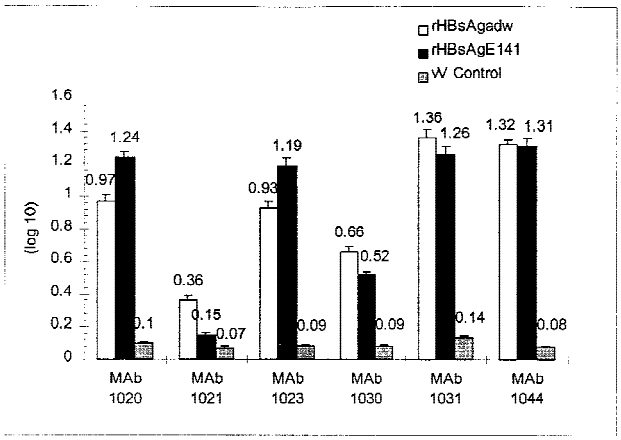
tope between amino acids 139 and 147 has been previously shown as binding to the *a* determinant (Waters et al., 1991), thus confirming that the change of amino acid from lysine to glutamic acid at position 141 (Fig. 1b) affected one of the *a* determinant epitopes.

Comparative Reactivity for Plasma-Derived (*adw*) and Recombinant (*ayw4*) HBsAg Particles

Initially, the panel of six monoclonal antibodies (1020, 1021, 1023, 1030, 1031, and 1044) were titrated against plasma-derived HBsAg, subtype *adw* in order to measure the range of antibody concentrations present against HBsAg of the immunising subtype and to select a suitable dilution for further studies. All antibodies produced titres spanning the range $\log_{-10}$ 4.7 to $\log_{-10}$ 6.0. The dilution curves were similar, suggesting that the relative affinities for the antigen substrate were comparable (data not shown). These data were used to standardise the antibody dilutions required for subsequent assays.

The panel of monoclonal antibodies was compared for reactivity using antigen of the same subtype used as immunising antigen and HBsAg particles of the *ayw4* subtype prevalent in West Africa. The antibodies were diluted to $\log_{10}$ 5.1 and assayed against subtype *adw* HBsAg by ELISA (Table I). Three antibodies (1030, 1031, and 1044) produced statistically comparable reactivities. However, the remaining antibodies gave values which were significantly lower ($P \leq .034$) but were also significantly different to each other ($P < .001$). Substitution of the antigen substrate with the heterologous (*ayw4*) antigen produced using the cellular ELISA resulted in a significantly different pattern of reactivity. The differences between antibodies 1031 and 1044 were not significant, whereas the differences between the two antibodies and the remaining four were highly significant ($P < .001$) as were the differences between them ($P \leq .017$). These differences are seen more clearly as a ratio of mean $OD_{490\ nm}$ values expressed relative to the $OD_{490\ nm}$ value obtained with antibody 1044 (data not shown). Thus the relative reactivities of the antibodies 1031 and 1044 were comparable, confirming that these reagents recognised an epitope in the *a* determinant common to both HBV genotypes B and E. In contrast, the remaining four antibodies showed marked differences, with antibody 1021 showing the greatest discrimination between the two antigens.

(a)



(b)

$\log_{10}$ dilution	2.9	2.6	3.5	3.5	4.1	2.9
p value	<0.001	<0.001	<0.001	<0.001	<0.001	NS[1]

Fig. 2. **(a)** Mean $OD_{490\ nm}$ values obtained with 5 to 6 replicates using recombinant antigens. **(b)** Antibody dilutions used in the analysis are indicated in parenthesis, SD was less than 0.05. The significance of the differences in the mean $OD_{490\ nm}$ of the two recombinant antigens for each of the antibodies was measured using the unpaired, single-tailed Student's *t* test, and expressed as probability values. NS, not significant.

Analysis of HBsAg Variant ($HBsAg^{E141}$) Using Cellular ELISA

Both recombinant vaccinia viruses ($vHBsAg^{ayw4}$ and $vHBsAg^{E141}$) expressed HBsAg in Vero cells as detected by immunoperoxidase staining using a 1:1000 dilution of the anti-HBs monoclonal antibody 1030 (data not shown). The staining was localised in the cytoplasm and at the cell membranes. There was no specific staining of cells infected with the control vaccinia virus lacking an S gene insert.

The contributions of the subtype determinant and/or the lysine to glutamic acid mutation at position 141 to the epitopes recognised by the six monoclonal antibodies were then measured against HBsAg expressed by the two vaccinia constructs ($vHBsAg^{ayw4}$ and $vHBsAg^{E141}$). Antibody binding could be demonstrated to both recombinant antigens using all six reagents (Fig. 2). No significant binding was observed using the vaccinia vector (vV) control. All antibodies were assayed using a minimum of five replicate dilution series for each antibody. The SD of the observed absorbance values was less than 0.05. These experiments were repeated on at least two further occasions and the results found to be reproducible.

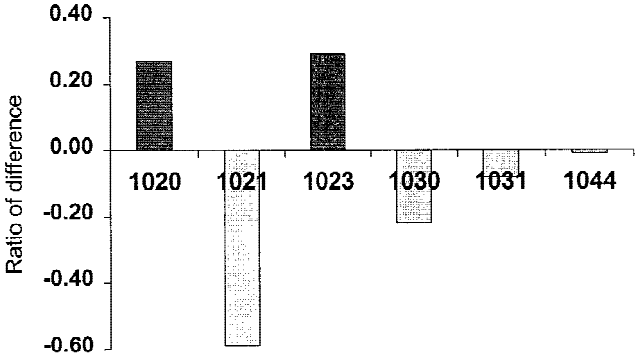


Fig. 3. Ratio of differences observed by ELISA between recombinant antigens HBsAg^{ayw4} and HBsAg^{E141}.

The mean values obtained showed differences in reactivity for the wild-type (HBsAg^{ayw4}) and variant antigens (HBsAg^{E141}). Differences in reactivity of the antibodies was particularly significant for the antibodies 1030 and 1021 ($P < .001$; Fig. 2). Antibody 1030 gave a higher reactivity with rHBsAg^{ayw4} ($OD_{490\ nm}$ of 0.66) compared to the reaction with rHBsAg^{E141} ($OD_{490\ nm}$ of 0.52). Antibody 1021, while having a low level reactivity with rHBsAg^{ayw4} of 0.36 $OD_{490\ nm}$, did not appear to recognise rHBsAg^{E141}; assuming a cutoff value of $OD_{490\ nm}$ of the negative control plus two SD units, the minimum positive value was 0.19, much higher than the observed mean $OD_{490\ nm}$ of 0.15 with rHBsAg^{E141} as the substrate ($P < .001$). By contrast, there appeared to be no difference in reactivity of antibody 1044 for the two recombinant antigens. The two antibodies 1020 and 1023 showed a more limited difference in reactivity. Antibody 1020 gave an $OD_{490\ nm}$ of 1.24 when reacted with rHBsAg^{E141} and an $OD_{490\ nm}$ of 0.97 on reaction with rHBsAg^{ayw4}. The remaining antibody, 1023, was similarly higher in reactivity for rHBsAg^{E141} ($OD_{490\ nm}$ 1.19) than rHBsAg^{ayw4} ($OD_{490\ nm}$ 0.93).

The ratio of the differences in reactivity of the two antigens was expressed as a ratio of reactivity with rHBsAg^{ayw4}. Antibody 1021 displayed the most marked difference with a ratio of −0.59 compared with the ratio of −0.01 detected with antibody 1044 (Fig. 3). The relative ratios obtained with the remaining four antibodies varied from −0.08 to +0.29.

DISCUSSION

The antibody response to the outer coat of HBV is largely directed to antigenic determinants located between amino acids 120 and 150 of the major S protein, the predominant component of HBsAg particles. Many studies in recent years have reported that variants of HBV may arise, particularly in children administered both hepatitis B vaccine and hepatitis B-specific immunoglobulin (HBIG). The most intensively studied of these changes is at position 145, manifested by substitution of arginine for glycine. Other changes have been recorded less frequently, the majority being clustered between residues 140 and 150 (Howard et al., 1995b).

Breakthrough infections among immunised children in The Gambia have been associated with a point mutation at nucleotide 421, resulting in an amino acid substitution of glutamic acid for lysine at position 141 in the 226-residue S protein. In contrast to other studies, these children received vaccine only, which may account for the appearance of this unique change in approximately 8% of immunised children vaccinated as part of a nationwide immunisation programme (Howard et al., 1995b). The S gene sequence of the classical strain of HBV which circulates in The Gambia is of the *ayw4* subtype belonging to the genotype Group E whilst the strain used in most plasma-derived vaccines is of the *adw* subtype belonging instead to Group A (Norder et al., 1992). This study demonstrates that there are distinctive antigenic differences between the vaccine-strain of HBsAg of the *adw* subtype, and the HBsAg of the *ayw4* subtype circulating in West Africa. A distinct difference in the antigenicity of these two antigens is attributable to *d/y* genotype (Okamoto et al., 1989) encoded at position 122. HBsAg^{adw} encodes lysine at this position whilst rHBsAg^{ayw4} encodes arginine. Two additional amino acid difference of significance were located at positions 127 and 131 of the S sequence. The proline and asparagine encoded at codon 127 and 131 respectively in Group A subtypes is replaced by leucine and threonine in Group E (Fig. 1b). These could contribute to the difference in the antigenicity observed in this study.

The lysine 141 to glutamic acid substitution represents the only difference in the amino acid sequences of the S protein of the two Gambian strains within the surface domain between amino acids 101 and 159 (Stirk et al., 1992). The replacement of lysine 141 by glutamic acid in the variant strain results in a residue with negative polarity in close proximity with aspartic acid 144, which is also of negative polarity. This may have a significant effect on the secondary structure within the polyprotein in particular as the residues are brought into close proximity by a β-turn between proline 142 and upstream of asparagine 146 (Stirk et al., 1992). Thus, perturbations in the secondary structure of the surface protein of rHBsAg^{E141} caused by the substitution of glutamic acid for lysine 141 possessed by the rHBsAg^{ayw4} may affect both group and subtype antigenic determinants of HBsAg.

In summary, the six antibodies used in this study demonstrate specific patterns of reactivity with all three sources of HBsAg used in this study (Table I). Antibody 1044, which probably recognises a group specific epitope on HBsAg^{adw}, was equally reactive with the rHBsAg^{ayw4} and rHBsAg^{E141}, illustrating that this reagent recognises an epitope outside of the domain which includes amino acid 141. Antibody 1021, and to a lesser extent antibody 1030, effectively differentiated the two preparations HBsAg^{adw} and rHBsAg^{ayw4}. Antibodies 1021, 1020 and 1023 displayed the most marked differences in reactivity for the rHBsAg^{ayw4} and rHBsAg^{E141} particles.

Other studies have demonstrated the importance of lysine 141 for the maintenance of the antigenicity of

the S protein. Amino acid replacement studies have examined the effect of substituting each amino acid in the S protein by each of the remaining 19 possible amino acids on the reactivity to rabbit anti-HBs antibodies; these have confirmed the immunodominance of amino acids 141 to 145 (Steward et al., 1993). Replacing the lysine by any of the other amino acids including glutamic acid abolished the reactivity. A similar reduction in the reactivity for rabbit anti-HBs was detected when lysine 141 was replaced by glutamic acid, glycine, alanine, serine, aspartic acid, or arginine (Neurath et al., 1990).

The significantly higher reactivity of antibodies 1020 and 1023 with rHBsAg^{E141} than with rHBsAg^{ayw4}, and the converse with antibodies 1021, 1030, and 1031, confirms that the two isolates from The Gambia are antigenically different. The antigenic determinants affected were probably both group-specific and subtype-specific. In particular, the reduced binding of rHBsAg^{E141} to antibody 1031, which has been shown to recognise the group-specific *a* determinant, shows that the change of the lysine residue at position 141 for glutamic acid has altered the antigenicity at this site, most probably by inducing a significant conformational change in the second of the two predicted hydrophilic loops (Stirk et al., 1992). These differences may account for the failure to detect HBsAg, an important serological marker of HBV infection, in the two children with breakthrough infections reported previously (Karthigesu et al., 1994). The differences in the antigenicity of the Gambian variant strain and the vaccine strain suggest that vaccinated individuals in The Gambia may still become infected with HBV. It remains to be determined, however, whether or not the emergence of this or other variants plays a significant role in the epidemiology of HBV in The Gambia and elsewhere. Cumulative data from two immunised cohorts in The Gambia have shown that the number of breakthrough infections is less than initially predicted and has not adversely affected the decline in HBV carriage among immunised individuals. Notwithstanding this, it may be prudent to configure diagnostic tests that take into account HBV variants that give rise to phenotypically and antigenically distinct HBsAg particles in order to detect individuals carrying potentially infectious variants of HBV.

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